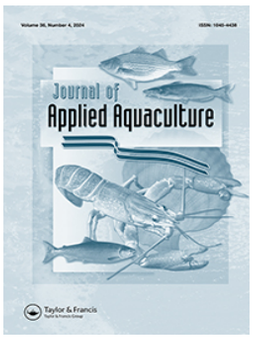

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Exploring the potential of tiger milk mushroom (*Lignosus rhinocerotis*) crude extracts as immunostimulants for Pacific white shrimp (*Litopenaeus vannamei*): effects on survival and immunological responses against *Vibrio parahaemolyticus*

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ABSTRACT

This study investigated the effects of tiger milk mushroom (*Lignosus rhinocerotis*) extract (LRE) on immune responses and resistance against the aquatic pathogen *Vibrio parahaemolyticus* in Pacific white shrimp (*Litopenaeus vannamei*) to improve disease management in shrimp aquaculture. LREs were extracted using methanol and water to evaluate their cytotoxicity and antimicrobial efficacy. *L. rhinocerotis* methanol extract (LRME) exhibited higher antimicrobial activity and lower acute toxicity compared to the aqueous extract, and was selected for subsequent in vitro hemocyte assays and in vivo feeding trials. The in vivo trials were conducted in two batches, with each batch consisting of five diets: a basal diet without LRME inclusion (control), a commercial diet, and three treatment diets supplemented with LRME at 1 g (LRME1), 5 g (LRME5), and 10 g (LRME10) per kilogram of feed. Each diet had three replicates ($n = 3$). The first batch included 120 shrimp with an average weight of 13.38 ± 1.72 g, and was used to evaluate immune parameters, including total hemocyte count (THC), hemocyte type proportions, phenoloxidase (PO) activity, phagocytic activity, and reactive oxygen species (ROS) production, over a 12-day feeding trial. The second batch consisted of 225 post-larvae

KEYWORDS

Bacterial disease; disease resistance; fungal feed additives; Immunostimulant; shrimp immunity

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This article has been corrected with minor changes. These changes do not impact the academic content of the article.

(PL10) with an average weight of 0.06 ± 0.01 g, and was used for bacterial challenge tests to assess survival rates following a 4-day feeding trial. In the 12-day feeding trial, shrimp fed with LRME showed significant increases in THC, ROS production, phagocytic, and PO activity, with LRME1 consistently demonstrating substantial improvements across multiple immune parameters. Days 4 and 8 were observed as specific time points when LRME exhibited significant effects on shrimp immunity, highlighting the temporal nature of shrimp immune responses to LRME supplementation. In a subsequent bacterial challenge test, a 4-day supplementation of LRME1 significantly enhanced shrimp survival rates. This study highlights the potential of LRME in enhancing shrimp immunity and resistance against *V. parahaemolyticus*, with significant effects observed at 1 g kg^{-1} dietary inclusion and a supplementation period of 4 days.

Introduction

Pacific white shrimp (*Litopenaeus vannamei*) holds significant commercial importance in aquaculture and is extensively farmed globally, accounting for approximately 80% of the total production of cultured penaeid shrimp (Zhang et al. 2019). However, the emergence of acute hepatopancreatic necrosis disease (AHPND), characterized by significant hepatopancreas atrophy and distinct histopathological changes during the acute phase of infection, has led to significant economic losses, reaching billions of dollars annually since the initial outbreak in China in 2009, subsequently affecting other Southeast Asian countries, such as Vietnam (2010), Malaysia (2011), Thailand (2012), the Philippines (2013), and Mexico (2013) (De la Peña et al. 2015; Nunan et al. 2014; Soto-Rodriguez et al. 2015; Tran et al. 2013).

Vibrio parahaemolyticus, identified as the causative agent of AHPND, is a gram-negative bacterium with a rod-shaped morphology that is widely present in the natural environment (Sirikharin et al. 2015). When the natural defense mechanisms of the host are suppressed, they can become opportunistic pathogens that can infect both human and aquatic animals. The species' common virulence determinants in shrimp are the binary toxins $pirA^{vp}$ and $pirB^{vp}$ (Han et al. 2015). The mechanism of infection involves the bacterium colonizing shrimp intestines, releasing binary toxins, and causing massive cell sloughing in the hepatopancreas (Lai et al. 2015). Shrimps, lacking specific immunity, face challenges in combating these pathogens. The use of chemicals and antibiotics in aquaculture to manage disease outbreaks has raised concerns about the development of antibiotic resistance, which poses a threat to human and animal health as well as the environment (Holmstrom et al. 2003).

Natural products, with immunomodulatory and antimicrobial properties have emerged as viable solutions, with fungal derivatives demonstrating promising results in enhancing innate immune responses in

aquatic organisms (Dashtiannasab et al. 2016; Militz and Hutson 2015; Shefat 2018). Mushroom and fungal derivatives (glucan, nucleotide, vitamins, chitin, protein etc.) act as immunostimulants, enhancing shrimp immunity by promoting the growth of intestinal gut microbiota, activating the phagocytic cells and prophenoloxidase (proPO) system, and boosting the antibacterial properties in the hemolymph (Sang, Kien, and Thanh Thuy 2014; Wilson et al. 2015; Mohan et al. 2019; Ernesto; Ceseña et al. 2021).

Among these, tiger milk mushroom (*Lignosus rhinocerotis*), stands out due to its well-documented medicinal properties, high availability and diverse bioactive compounds (Nallathamby et al. 2018). The immunomodulatory properties of *L. rhinocerotis* have been linked to its ability to activate innate immune cells and T-helper cells, suggesting a potential role in regulating immune responses (Wong, Lai, and Cheung 2011). Nallathamby et al. (2016) demonstrated the potential of *L. rhinocerotis* to modulate inflammatory responses by reducing nitric oxide/inducible nitric oxide synthase (NO/iNOS) and cyclooxygenase-2 (COX-2) proinflammatory genes and via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the signal transducer and activator of transcription 3 (STAT3) pathway. Eik et al. (2012) discovered that an aqueous extract derived from *L. rhinocerotis* sclerotium contains NGF-like compounds that enhance neurite outgrowth activity. These compounds could activate the p38 mitogen-activated protein kinase (MAPK) pathway, a crucial innate immune pathway in shrimps involved in pathogen invasion as highlighted in studies by He et al. (2013) and Yan et al. (2013). Moreover, the antimicrobial activity of *L. rhinocerotis* has been observed against various human pathogens, with studies identifying active phytochemical constituents, such as steroids, terpenoids, alkaloids, and flavonoids (Kamal, Adnan, and Latip 2023). Mohanarji, Dharmalingam, and Kalusalingam (2012). demonstrated the antimicrobial activity of *L. rhinocerotis* against *Vibrio cholera*, suggesting its potential efficacy against other Vibrio species, such as *V. parahaemolyticus*.

While research has demonstrated the dual potential of *L. rhinocerotis* in activating shrimp immune pathways and exhibiting antimicrobial activity against *Vibrio* species, its specific impact on the immune responses of shrimp remains an underexplored domain. Therefore, this study seeks to address this gap by investigating the dietary supplementation of *L. rhinocerotis* extract (LRE) on the immunomodulatory effects of *L. vannamei*. Specifically, it evaluates immune parameters, including hemocyte counts, phagocytic activity, PO activity, ROS production, and survival rates following bacterial challenge with *V. parahaemolyticus*. The findings aim to contribute to the integration of natural compounds, such as LRE, into aquaculture feeding programs to improve shrimp health and disease management.

Materials and methods

Biological materials and pathogen culture

Lignosus rhinocerotis sclerotial powder (TM02) was obtained from LiGNO Biotech Sdn. Bhd. (Balakong Jaya, Selangor, Malaysia). The virulent strain of *Vibrio parahaemolyticus* was acquired from University Putra Malaysia, Serdang, Selangor, Malaysia. Pacific white shrimp (*Litopenaeus vannamei*) were obtained from Hilex Aquatic Sdn. Bhd., located in Jeram, Selangor, Malaysia.

V. parahaemolyticus was cultured and maintained in Luria-Bertani (LB) broth supplemented with 1.5% NaCl and thiosulfate citrate bile sucrose (TCBS) agar media. For experimental use, fresh cultures were prepared by subculturing overnight cultures in fresh LB broth at a 1:100 ratio for 4 h to reach mid-log phase (OD 0.85, 1.5×10^8 CFU mL^{-1}). After centrifugation at 10,000 rpm for 10 min at 25°C (Elshopakey et al. 2018), bacterial pellets were resuspended and adjusted using sterile saline (0.9% NaCl) to the desired concentration.

Preparation of L. rhinocerotis extracts (LREs)

Lignosus rhinocerotis extracts (LREs) were prepared following the method described by Mohanarji, Dharmalingam, and Kalusalingam (2012). To compare the effectiveness of different solvents in extracting bioactive compounds, 100 g of sclerotial powder was mixed with 500 mL of either methanol or distilled water to produce *L. rhinocerotis* methanol extract (LRME) and aqueous extract (LRAE), respectively (Figure 1). Each mixture was stirred in a shaking incubator (IKA-Werke, Germany) at 30°C at 150 revolutions per minute (rpm) for 24 h, filtered through Whatman filter papers, and concentrated using a rotary vacuum evaporator (*R*-200, Büchi, Switzerland) at 40°C under reduced pressure (97.4 kPa). Extracts prepared using the same solvent were pooled, forming separate methanol and aqueous extracts, and stored in a desiccator until further use.

Cytotoxicity and antimicrobial assays

The cytotoxicity of LREs was assessed using brine shrimp (*Artemia salina*) lethality tests as described by Niksic et al. (2021), and antimicrobial activity against *V. parahaemolyticus* was tested using broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines (2012). Median lethal concentration (LC_{50}), minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of each tested compound were determined. Results indicated LRME exhibited higher antimicrobial activity and lower cytotoxicity compared to LRAE.

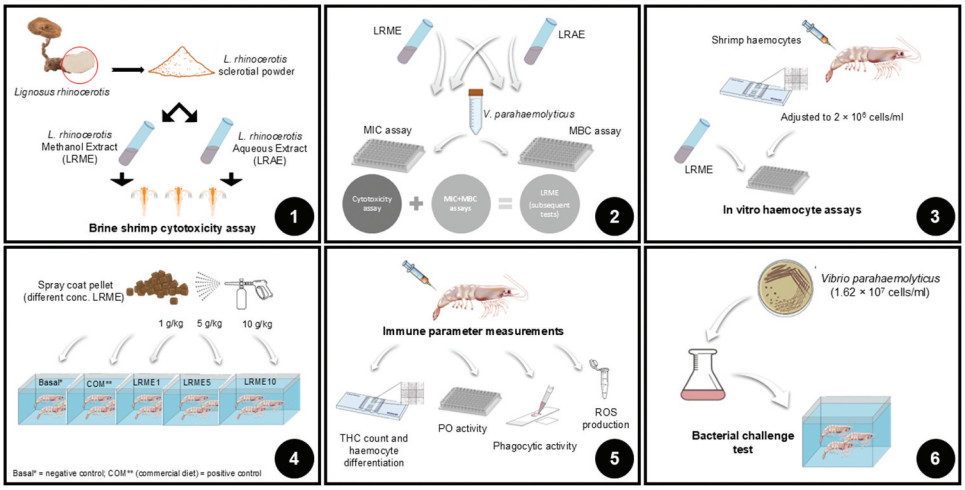

Figure 1. A schematic overview of the experimental design, including the preparation of *lignosus rhinocerotis* extracts, brine shrimp cytotoxicity assay, antimicrobial assays, in vitro haemocyte assays, in vivo feeding trials, immune parameter measurements, and bacterial challenge tests.

Haemocyte viability assay

LRME was selected for further testing due to its higher antimicrobial effect and lower acute cytotoxicity. Different concentrations of LRME (0, 0.01, 0.10, 1.00, and 10.00 mg mL^{-1}) were prepared to assess their effects on shrimp hemocyte viability, with three replicates per concentration. Haemolymph was collected aseptically from the ventral sinus of shrimps (11–13 g) using a 25-gauge needle with a 1-mL syringe containing anticoagulant buffer (30 mm trisodium citrate, 0.34 M NaCl, 10 mm ethylenediaminetetraacetic acid (EDTA), and 0.12 M glucose) (Dewi et al. 2021). Hemocyte concentrations were adjusted to 2×10^6 cells mL^{-1} with anticoagulant buffer.

The assay was performed by evaluating the reduction of tetrazolium salts (MTT) in shrimp hemocytes (Domínguez-Borbor, Chalén-Alvarado, and Rodríguez 2018). Haemocyte suspensions (2×10^6 cells mL^{-1}) were pipetted into 96-well plates (100 µL/well) and incubated for 1 h at room temperature for adherence. Following incubation, the supernatant was removed and incubated with 100 µL of LRME solution for 90 min at 25°C. Then, 10 µL of 5 mg mL^{-1} MTT was added and further incubated for 120 min at 25°C in the dark. Subsequently, 150 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals, and absorbance was measured at 590 nm. The hemocyte viability percentage was determined in comparison with the non-stimulated treatment (0 mg mL^{-1}):

$$\text{Cell viability}(\%) = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100\%$$

Experimental feed preparation

The supplementary dosages for the *in vivo* experiment were chosen based on their effects on cell viability. The highest concentration (0.10 mg mL^{-1}) that did not significantly impact hemocyte viability, and the lowest concentration (1.00 mg mL^{-1}) that significantly decreased cell viability and an intermediate concentration (0.50 mg mL^{-1}) were selected. To account for potential dilution or degradation of active compounds during the feeding process, a factor of 10 times these doses was applied to determine the dietary dosage. These concentrations were converted to dietary dosages of 1 g (LRME1), 5 g (LRME5), and 10 g (LRME10) of LRME per kg of feed, respectively (Figure 1). Along with a basal diet (without LRME) and a commercial diet (SMART #4, Grobest Malaysia, 37% crude protein, 5% crude fat, 4% fiber) served as a comparative reference, five experimental diets were included in the *in vivo* feeding trials. A commercial diet (SMART #4) with specific features to enhance overall health and disease resistance, served as a comparative reference. The basal diet was prepared using ingredients as outlined in Table 1. Feed ingredients were weighed and mixed thoroughly. Fish oil, vitamins, and mineral mix were added to the dry mixture, followed by stirring until the ingredients were evenly mixed. The feed mixture was pelleted into 6-mm-long pellets using a pelleting machine (Model 160, Guangzhou Juncheng Machinery Equipment Co. Ltd, China).

Treatment diets were prepared by applying LRME onto the basal diet feed pellets as a spray coat to prevent thermal deactivation of bioactive compounds during pelleting, as described by Wang, Lin, and Zhong (2021) with slight modifications (Figure 1). LRME was weighed and dissolved in 200 mL of deionized water in a spray bottle to achieve the desired concentrations for LRME1, LRME5, and LRME10 diets, respectively. Basal feed pellets were spread as a single layer on a stainless-steel tray. For every 1 kg of basal feed, 200 mL of the respective LRME solution was sprayed onto the feed pellets using a spray bottle.

Shrimp feeding trial

Two batches of Pacific white shrimp (*Litopenaeus vannamei*) were used in this study: Batch 1, consisting of shrimp weighing approximately 13.38 ± 1.72 g), and Batch 2, consisting of post-larvae (PL 10) weighing approximately 0.06 ± 0.01 g. Batch 1 was selected for the immune parameter measurements due to the larger size, which allowed for sufficient hemolymph collection. Batch 2 was used exclusively in the bacterial challenge test, as AHPND generally affects shrimp during the PL stage.

The shrimp were acclimated in 50 L glass tanks for one week and fed twice daily at 10% of their body weight with a commercial diet (BLANCA 7701,

Table 1. Diet formulation for basal diet.

Ingredient (g kg^{-1})	
Fishmeal	660.0
Wheat flour	124.0
Alginate (seaweed extract)	40.0
Squid liver oil	45.0
Fish oil	30.0
Soybean oil	20.0
Palm kernel cake (PKC)	20.0
Lecithin	5.0
Mineral mixture[a]	25.0
Vitamin mixture [b]	15.0
Antioxidant	0.5
Choline chloride	1.0
Vitamin C	1.0
Monocalcium phosphate (MCP)	1.0
Lysine	5.0
Methionine	5.0
Anti-fungal agent	2.5
Proximate nutrient content (g kg^{-1})	
Crude protein	452 ± 1
Crude fat	95 ± 1
Total carbohydrate	190 ± 2
Ash	144 ± 1
Moisture	119 ± 1

[a]One kilogram of mineral mixture contained the following: Zn 30.00 g, Mn 20.00 g, Fe 80.00 g, copper 4.000 g, Iodine 1.500 g, Selenium 0.125 g, Cobalt 0.150 g, Magnesium 50.00 g.

[b]One kilogram of vitamin premix contained the following: Vit A 20.000 MIU, Vit D3 4.000 MIU, Vit E 52.000 g, Vit K3 4.000 g, Vit B1 4.000 g, Vit B2 10.000 g, Vit B6 6.400 g, Vit B12 40.000 g, Pantothenic acid 22.40 g, Nicotinic acid 80.00 g, Folic acid 3.20 g, Biotin 200.00 g, Phytase 10,000.00 FTU.

Charoen Pokphand Foods Malaysia) with a pellet diameter of 0.5–1.0 mm and nutrient composition of 36% crude protein, 5% fat, and 4% fiber. Water quality was maintained at 26.0 ± 0.5°C, pH 8.0 ± 0.3, salinity 15.0 ± 1.0 ppt, and dissolved oxygen 5.0 ± 1.0 mg mL^{-1}. Ammonia and nitrate levels were maintained at 0.25 mg L^{-1} and 5.0 mg L^{-1}, respectively, using API ammonia and nitrate test kits (Mars, Inc., USA). The tanks were maintained under constant aeration, and 50% of the total water volume was changed twice a week.

The experimental design consisted of five diets: a basal diet without LRME inclusion (control), commercial diet (COM), LRME1, LRME5, and LRME10, each with three replicates ($n = 3$). Throughout the feeding trials, the shrimps were fed twice daily at approximately 10% of their body weight. Uneaten feeds were collected, and feces were siphoned out daily.

For immune parameter measurements, including total hemocyte count (THC), the proportion of different hemocyte types, phenoloxidase (PO) activity, phagocytic activity, and Reactive oxygen species (ROS) production, a total of 120 shrimp (Batch 1) were distributed into 15 tanks, and divided among five

diet groups with three replicates each. These immune parameters were assessed at four time points: days 0, 4, 8, and 12, with 100 µL of hemolymph collected from each individual shrimp ($n = 3$ per treatment), as described in Section 2.4.

Total haemocyte count (THC) and haemocyte differentiation

Ten microliters of the anticoagulant-hemolymph mixture were dispensed onto a hemocytometer (Neubauer Improved, Assistent, Germany) for THC determination (Dewi et al. 2021). The sample was observed under a microscope at 400 × magnification and the THC was calculated as follows:

$$\mathrm{THC}\left(\frac{\mathrm{cells}}{\mathrm{mL}}\right) = \text{number of cells in a}1\,\mathrm{m\,m^2}\ \text{square} \times 10^4\,\mathrm{mL} \times \text{ dilution factor}$$

Subsequently, the numbers and relative proportions of distinct hemocyte types, namely hyaline cell (HC), semi-granular cell (SGC), and granular cell (GC), were determined by counting a minimum of 200 cells under a microscope. The percentage of each hemocyte cell type was calculated as follows:

$$\text{Haemocyte percentage}(\%) = \frac{\text{number of differential haemocyte cell type}}{200} \times 100$$

Phenoloxidase (PO) activity of shrimp haemolymph

PO activity was measured by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) (Nacalai tesque, Japan) using 96-well microplates as described by Noble et al. (2023) with slight modifications. The hemolymph-anticoagulant mixture (2×10^6 cells $\mathrm{mL^{-1}}$) was centrifuged at 800 × g at 4°C for 20 min. The supernatant was removed, and the cells were resuspended in the same volume of Tris-HCl buffer. One hundred microliters of hemocyte suspension were added to each well of a 96-well plate containing 100 µL of L-DOPA (3 mg $\mathrm{mL^{-1}}$ in Tris-HCl buffer). Blank control solutions were prepared using the same volume and reagents, with the hemocyte suspension replaced by Tris-HCl buffer. These control solutions were included to account for the spontaneous oxidation of the substrate alone for each concentration tested (Smith and Söderhäll 1991). The hemocyte suspensions were measured after 15 min of reaction time at 490 nm using a microplate reader (FLUOstar Omega, BMG Labtech, Germany). PO activity is presented as absorbance relative to the blank control (Dewi et al. 2021) as follows:

$$\text{PO activity} = \text{OD of sample} - \text{OD of blank control}$$

Phagocytic activity of shrimp haemolymph

Phagocytic activity was measured according to a previously described method (Dewi et al. 2021) with slight modifications. One hundred microliters of hemocyte suspension (2×10^6 cells mL^{-1}) were dropped on a glass slide and left for 60 min at room temperature for cell adherence. Subsequently, the cells were washed with PBS to eliminate any remaining unattached cells; then, 100 µL of *V. parahaemolyticus* (~10^6 CFU mL^{-1}) were added. The cells were washed with PBS after 30 min of incubation, fixed with methanol for 5 min, stained with 6% Giemsa for 20 min, and then decolorized with distilled water. Subsequently, they were air-dried and observed using a light microscope under 100× magnification. Phagocytic activity was calculated as the percentage of hemocytes containing bacteria within the cytoplasm among 200 hemocytes, using the following formula:

$$\text{Phagocytic activity}(\%) = \frac{\text{phagocytic haemocytes}}{200} \times 100$$

Reactive oxygen species (ROS) production of shrimp haemolymph

ROS levels were measured in shrimp hemocytes using a fluorometric kit (Cat. No. E-BC-K138-F, Elabscience, USA) that measures the fluorescent absorbance of 2′,7′-dichlorofluorescein (DCF) as described by Elshopakey et al. (2018). One hundred microliters of hemocyte suspension (2×10^6 cells mL^{-1}) were incubated with DCFH-DA (final concentration at 0.5 mm) for 30 min at 37°C in the dark. The fluorescence reading was obtained at 485-nm excitation and 520-nm emission wavelengths using a microplate reader (Perera et al. 2021).

V. parahaemolyticus challenge test

A total of 225 shrimp PL 10 were distributed across 15 glass tanks. Each tank housed 15 shrimps, ranging from 0.05 g to 0.07 g, averaging 0.06 ± 0.01 g, with no significant initial weight differences among treatments. After 4 days of feeding, shrimps from each tank were subjected to a bacterial challenge with *V. parahaemolyticus* at a concentration of 1.62×10^7 CFU mL^{-1} (median lethal dosage, LD_{50} determined at 48 h) by immersion as described by Mazón-Suástegui et al. (2018). Three tanks of shrimp that had received a basal diet without exposure to bacteria served as the unchallenged control group. The mortality of shrimp in each tank was closely observed and recorded every 24 h for 120 h. The survival rate of each experimental group was determined (Figure 1).

Statistical analysis

One-way analysis of variance (ANOVA) was used to assess significant differences among treatment groups, followed by Tukey's HSD test ($p < .05$) for pairwise comparisons. Normality (Shapiro-Wilk) and homogeneity (Levene's) tests were performed beforehand. For data violating normality, such as MIC and MBC values, Kruskal-Wallis and Mann-Whitney U tests were applied. In vitro hemocyte viability data, with normal distribution but unequal variances, were analyzed using Welch ANOVA and Games-Howell post-hoc test. Immune parameters, including THC, hemocyte proportions, PO activity, ROS production, and phagocytic activity, were analyzed using ANOVA with normalized data and no violation of homogeneity. Survival rates following bacterial challenges were also analyzed with ANOVA and Tukey HSD. All analyses were performed using SPSS version 27 (IBM Corp., Armonk, NY, USA).

Results

Cytotoxicity and antibacterial activity of L. rhinocerotis extracts (LREs)

According to Clarkson's and Meyer's toxicity index, LRME exhibited a nontoxic profile with an LC_{50} of 1034.58 ± 98.13 μg mL^{-1} (Table 2), which was significantly higher ($p < .05$) than LRAE which had an LC_{50} of 386.23 ± 80.43 μg mL^{-1}. In the antimicrobial assays, LRME showed stronger antibacterial efficacy with lower MIC (8.00 ± 1.00 mg mL^{-1}) and significantly lower MBC (10.00 ± 0.00 mg mL^{-1}) compared with LRAE (MIC = 37.33 ± 1.53 mg mL^{-1}; MBC = 39.67 ± 0.58 mg mL), as shown in Table 3.

Effect of LRME on haemocyte viability in haemocytes

LRME did not exhibit significant toxicity at low concentrations (0.01 and 0.10 mg mL^{-1}) but it showed toxic effects at higher concentrations (1.00 and 10.00 mg mL^{-1}), as shown in Figure 2.

Table 2. Brine shrimp cytotoxicity assay results for LREs and control tetracycline HCl.

Test extract/compound	LC_{50} (μg mL^{-1}) (Mean ± SD)	Clarkson's toxicity index	Meyer's toxicity index
LRME	1034.58 ± 98.13[b]	Non-toxic	Non-toxic
LRAE	386.23 ± 80.43[a]	Moderately toxic	Toxic
Tetracycline HCl	1500.77 ± 182.36[c]	Non-toxic	Non-toxic

All values are presented as mean ± SD. Significant differences are denoted by different letters ($p < .05$) based on Tukey's HSD test at the 0.05 significance level. LRME, *L. rhinocerotis* methanol extract; LRAE, *L. rhinocerotis* aqueous extract.

Table 3. MIC and MBC of LREs against *V. parahaemolyticus*.

Type of extracts	MIC	MBC
LRME	8.00 ± 1.00 mg mL^{-1} ^{b}	10.00 ± 0.00 mg mL^{-1} ^{b}
LRAE	37.33 ± 1.53 mg mL^{-1} ^{b}	39.67 ± 0.58 mg mL^{-1} ^{c}
Tetracycline HCl (standard)	0.50 ± 0.00 μg mL^{-1} ^{a}	2.33 ± 1.53 μg mL^{-1} ^{a}

All values are presented as mean ± SD. Significant differences are denoted by different letters ($p < .05$) based on Mann-Whitney U tests at the 0.05 significance level. LRME, *L. rhinocerotis* methanol extract; LRAE, *L. rhinocerotis* aqueous extract.

Effects of dietary LRME on L. vannamei immunity responses

Supplementation with LRME in shrimp diets for 12 days induced immune system responses, including hematopoiesis. LRME supplementation modulated THC (Figure 3a), hemocyte differentiation (Figure 3(b,c,d)), PO activity (Figure 4a), ROS production (Figure 4b), and phagocytic activity (Figure 4c). LRME1 consistently demonstrated significant increase across various immune parameters, with distinct concentration-dependent effects.

Effects of dietary LRME on L. vannamei resistance to V. parahaemolyticus

In the 4-day feeding trial, LRME1 exhibited the highest SRs across all time points, significantly higher than all diet groups. LRME5 and the commercial diet followed, while LRME10 exhibited the lowest SRs across all time points (Figure 5). LRME5, LRME10, and the commercial diet did not statistically differ from the basal diet.

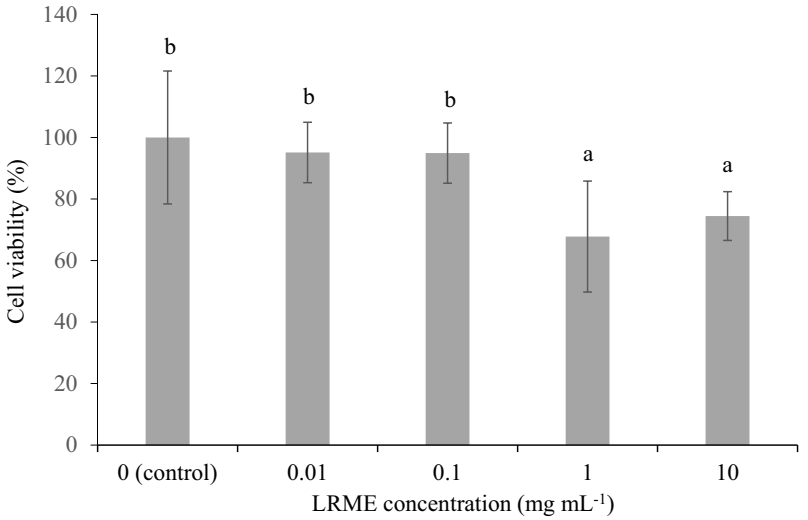

Figure 2. Cell viability of shrimp haemocytes following *in vitro* treatment with varying concentrations of LRME. Each bar represents the mean value with its corresponding standard deviation (SD). Statistically significant differences ($p < .05$) are indicated by different letters.

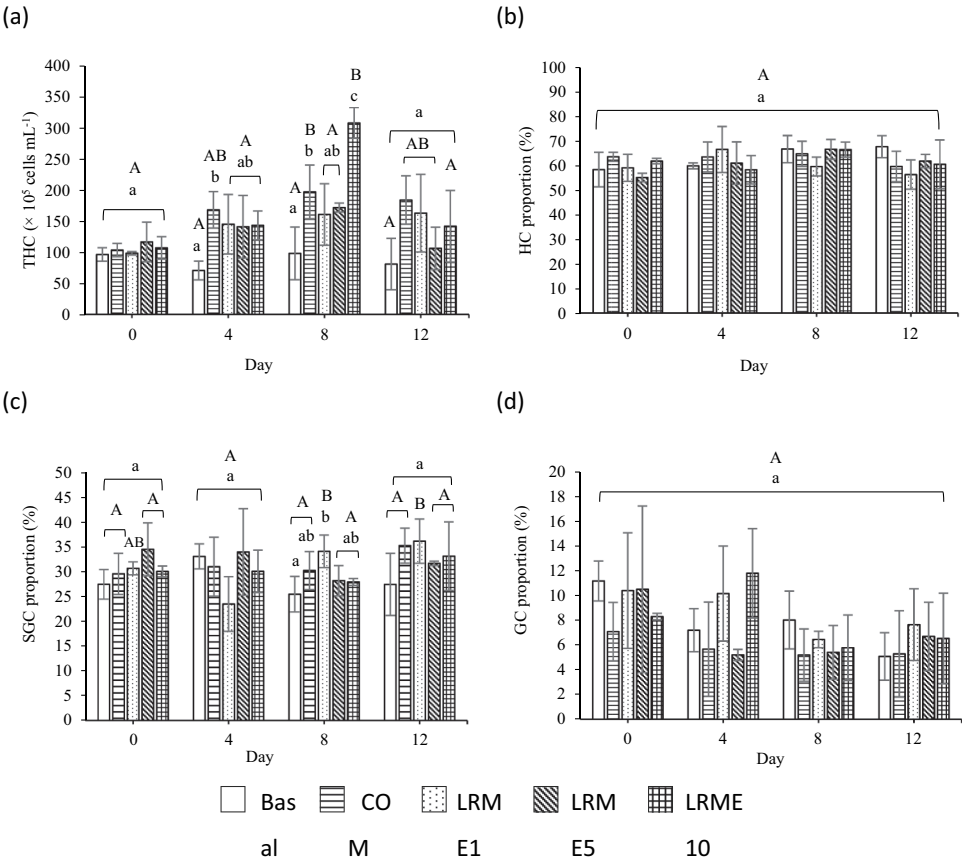

Figure 3. (a) THC, proportion of different haemocyte types: (b) HC, (c) SGC, and (d) GC in L. vannamei fed either a commercial shrimp diet (COM) or a developed diet supplemented with 0 (basal), 1, 5, or 10 g kg-1 of LRME over a 12-day feeding trial. Data are presented as mean ± SD (n = 3). Letters a, b, and c indicate significant differences among different diets on the same sampling day, whereas letters A and B indicate significant differences among different sampling days within the same diet, all of which were tested at a 5% level of significance in a post hoc Tukey's multiple comparison test.

Discussion

LREs exhibited notable antibacterial efficacy, with LRME showing significantly higher bacterial inhibitory effects and a nontoxic profile. Lau et al. (2014) identified several bioactive compounds in the sclerotial methanol extract of *L. rhinocerotis* that could have contributed to its potential benefits. These compounds, such as ergosterol and linoleic acid, are known for their antioxidant and antimicrobial properties, respectively (Dupont et al. 2021; Rangsinth et al. 2023; Yoon et al. 2018). Their presence likely explains the antimicrobial and immunostimulatory effects observed in the present study. LRME was selected over LRAE for incorporation into shrimp feed for *in vivo* experiments due to its higher bacterial inhibitory effects and lower cytotoxicity.

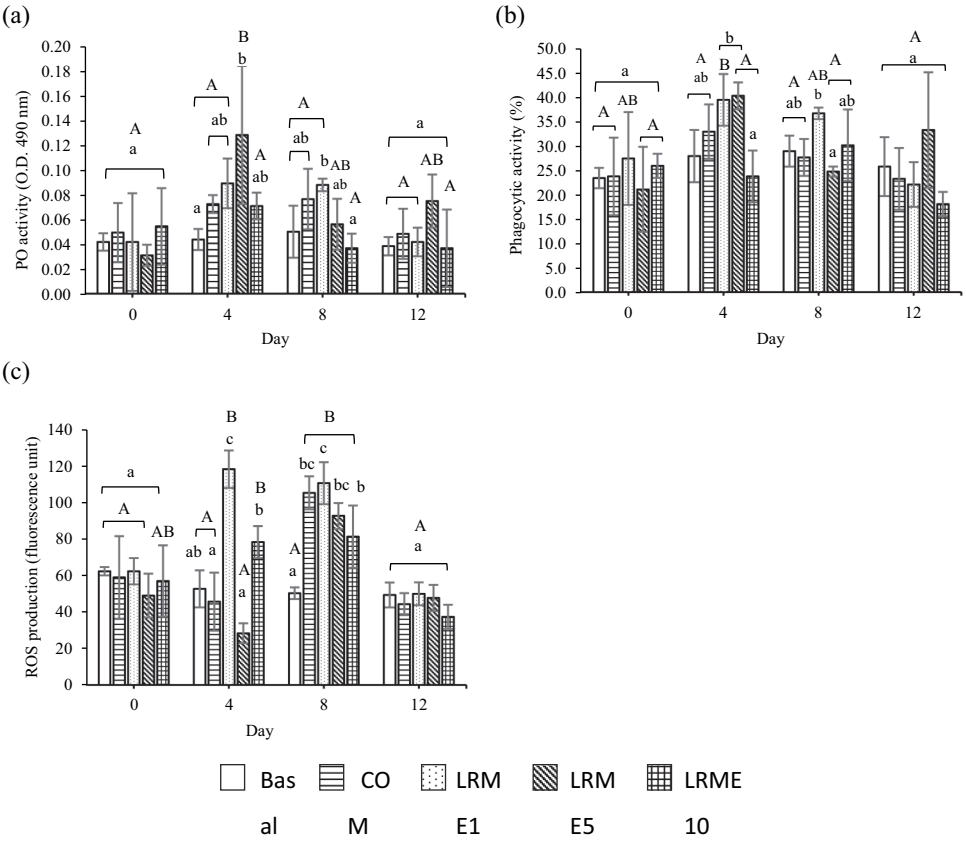

Figure 4. (a) PO activity (b) phagocytic activity (c) ROS production of L. vannamei fed either a commercial shrimp diet (COM) or a developed diet supplemented with 0 (basal), 1, 5, or 10 g kg-1 of LRME over a 12-day feeding trial. Data are presented as mean ± SD (n = 3). Letters a, b, and c indicate significant differences among different diets on the same sampling day, whereas letters A and B indicate significant differences among different sampling days within the same diet, all tested at a 5% level of significance in a post hoc Tukey's multiple comparison test.

The *in vivo* findings of this study highlight the immunomodulatory potential of LRME. Dietary incorporation of LRME significantly increased THC, hemocyte differentiation, PO activity, ROS production, and phagocytic activity of *L. vannamei*. The findings of the present study are consistent with those of previous research, such as the study by Ngo et al. (2020), which reported the immune-stimulating effects of polysaccharides and bioactive compounds in shrimp. Dewi et al. (2021) also observed similar results in *Penaeus vannamei*, where the incorporation of guava leaf extract into the diet led to elevated THC, PO activity, phagocytic activity, and upregulated expression of immune-related genes. The current study demonstrates that *L. rhinocerotis* – a species not previously studied for its effects on aquatic animal immunity – can serve as a novel source of immunostimulants.

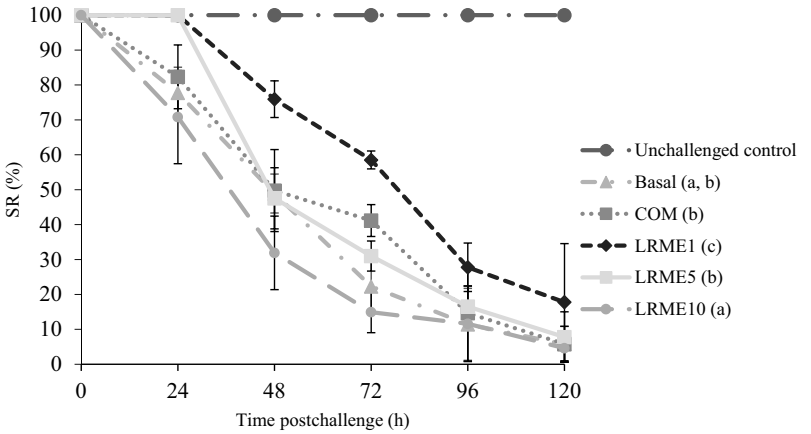

Figure 5. Survival of *L. vannamei* fed either a commercial shrimp diet (COM) or a developed diet supplemented with 0 (basal), 1, 5, or 10 g kg^{-1} of LRME challenged with *V. parahaemolyticus* for 120 h. Data are expressed as mean ± SD ($n = 3$), and significant differences ($p < .05$) are indicated with different letters.

Among the treatment groups, the lowest LRME concentration (LRME1), corresponding to 0.10 mg mL^{-1} in *in vitro* studies, consistently exhibited a significant increase compared with baseline activity across multiple immune parameters (Figures 3 and 4), without compromising cell viability (Figure 2). These results suggest that LRME1 is an ideal dosage for inclusion in shrimp diets to support immune function. However, higher doses (LRME5 and LRME10) did not enhance immune responses and were associated with lower immune parameters. This may be attributed to an overdose effect, where excessive LRME have had adverse effects on shrimp physiology and immune functions. Immunostimulants tend to exhibit optimal responses at intermediate concentrations, rather than following a linear dose–response relationship (Bliznakov and Adler 1972). At higher doses, the potential for immunosuppression or increased antioxidant activity may inhibit the immunostimulatory function of LRME, as reflected by reduced PO activities and ROS production in these groups.

Aligned with the immune parameter findings, the bacterial challenge results further demonstrated the impact of LRME dosage on shrimp resistance. Shrimp fed the LRME1 diet exhibited a significantly higher survival rate compared to those receiving higher LRME dosages (LRME5 and LRME10) and the control diet. Notably, the highest dosage of 10 g kg^{-1} (LRME10) even resulted in significantly reduced shrimp survival compared with the control group, indicating a threshold beyond which beneficial effects are lost.

The higher mortality rate observed in shrimp fed higher LRME dosages during the bacterial challenge could be attributed to exerted cytotoxic or immunosuppressive effects. This is supported by the *in vitro* hemocyte viability tests, which showed a significant decrease in cell viability beyond 0.10 mg mL^{-1}

(corresponding to the LRME1 dosage). Additionally, the 12-day feeding trial demonstrated diminished immunostimulatory activity at higher LRME dosages. While the extract may have initially influenced the immune response, but the resulting changes may not have been advantageous during the bacterial challenge, compromising the shrimp's ability to mount an effective response against bacteria (Sajeevan, Philip, and Bright 2009).

The lack of significant differences in immune parameters between days 0 and 12 across different diet groups, despite significant differences on days 4 and 8, suggests a temporal component of the immunostimulatory effects of LRME, with an initial activation followed by a return to baseline over the extended 12-day period. The observed dynamics possibly reflect the initial response and subsequent adaptation of shrimp hemocytes to LRME. This temporal aspect emphasizes the need for understanding the optimal doses and duration of LRME administration to avoid overstimulation and subsequent reversion to baseline immune function.

The commercial diet, specifically designed to promote shrimp health and disease resistance, showed a survival rate similar to that of the basal diet. However, the LRME1 diet outperformed the commercial diet with a significantly higher survival rate, suggesting that the immunomodulatory effects of the commercial diet may require a longer feeding period to manifest compared to the more immediate effects of the LRME1 diet. It is worth noting that the nutrient content of the commercial diet and the experimental diet differ, which makes the results case-specific. Further research with experimental diets containing nutrient content similar to the commercial diet could provide a more representative comparison.

Despite these considerations, with the basal diet without LRME serving as the baseline, this study demonstrates that the incorporation of LRME at 1 g kg^{-1} significantly enhanced shrimp resistance against *V. parahaemolyticus*.

With most studies focusing on its medicinal applications in human health, this study provides evidence for the immunomodulatory and antimicrobial potential of *Lignosus rhinocerotis* in aquaculture. LRME demonstrated significant antimicrobial activity against *Vibrio parahaemolyticus*, a common aquatic pathogen, suggesting its potential for pathogen control and disease management. Additionally, incorporating LRME into shrimp diets enhanced immune parameters and improved resistance to *V. parahaemolyticus*, offering a promising strategy for improving shrimp health in aquaculture.

Conclusion

In conclusion, this study establishes LRME as a promising antimicrobial agent against *V. parahaemolyticus* and an effective immunostimulant for enhancing shrimp health in aquaculture. Dietary supplementation of LRME at 1 g kg^{-1} enhanced shrimp immunity and resistance against the aquatic pathogen, outperforming a commercial diet with immune-enhancing properties during a 4-day feeding trial. However, higher doses did not provide additional benefits

and even reduced immune responses and survival, highlighting the need for further investigation into optimal dosing. Further investigations into the long-term effects of LRME supplementation and its mechanisms of action are essential to fully assess its potential for use in shrimp farming.

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Disclosure statement

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Authors' contributions

Q.E.P. contributed to the methodology, investigation, formal analysis, validation, data curation, and writing-original draft preparation. C.M.C. contributed to the methodology and data validation, P.T.L., Y.C., H.J.L. and K.P. contributed to manuscript review and editing. J.Y.L. and Y.C. contributed to funding acquisition and resources. J.Y.L. contributed to project administration, conceptualization, resources, supervision, and manuscript review and editing. The authors have read and agreed to submit this manuscript.

Ethics approval

No approval of the research ethics committees was required to accomplish the goals of this study because the experimental work was conducted with an unregulated invertebrate species.

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